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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : OUTTRUP, Helle [DK/DK]; Syvendehusvej 46, DK-2750 Ballerup (DK). DAMBMANN, Claus [DK/DK]; Høje Gladsaxe 61, 7. th., DK-2860 Søborg (DK). OLSEN, Arne, Agerlin [DK/DK]; Kaplevej 62, DK-2830 Virum (DK). BISGÅRD-FRANTZEN, Henrik [DK/DK]; Sandkrogen 27, DK-2800 Lyngby (DK). SCHÜLEIN, Martin [DK/DK]; Wiedeweltsgade 51, DK-2100 Copenhagen Ø (DK).

(74) Common Representative: NOVO NORDISK A/S; Patent Department, PeV, Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: ALKALOPHILIC *BACILLUS sp. AC13* AND PROTEASE, XYLANASE, CELLULASE OBTAINABLE THEREFROM

(57) Abstract

The present invention relates to novel microorganisms, novel enzymes obtainable herefrom, and to a method of producing the novel enzymes. More specifically, the invention relates to novel enzymes obtainable from strains of the novel alkalophilic species *Bacillus sp. AC13*. Moreover, the invention relates to a method for producing the enzymes of the invention, and to the use of the enzymes in detergents or in the paper pulp industry.

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Alkalophilic Bacillus sp. AC13 and protease, xylanase, cellulase obtainable therefrom.

TECHNICAL FIELD

The present invention relates to novel microorganisms, novel enzymes obtainable herefrom, and to a method of producing the novel enzymes. More specifically, the invention relates to novel enzymes obtainable from strains of the novel alkalophilic species Bacillus sp. AC13.

Moreover, the invention relates to a method for producing the enzymes of the invention, and to the use of the enzymes, particularly in detergents or in the paper pulp industry.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel microorganisms capable of expressing valuable novel enzymes for industrial applications, as well as novel enzymes of different kinds obtainable from these organisms.

Accordingly, the invention provides isolated biologically pure cultures of strains of the alkalophilic species Bacillus sp. AC13.

In another aspect, the invention provides enzymes obtainable from strains of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of an enzyme derived from Bacillus sp. AC13, NCIMB No. 40482.

In a more specific aspect, the invention provides proteases obtainable from strains of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a protease derived from Bacillus sp. AC13, NCIMB No. 40482.

In another specific aspect, the invention provides xylanases obtainable from strains of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a xylanase derived from Bacillus sp. AC13, NCIMB No. 40482.

In a third specific aspect, the invention provides cellulases obtainable from strains of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a cellulase derived from Bacillus sp. AC13, NCIMB No. 40482.

In a third aspect, the invention provides a process for the preparation of an enzyme of the invention, the process comprising cultivation of a strain of Bacillus sp. AC13, preferably the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme.

In further aspects, the invention provides detergent additives and detergent compositions comprising an enzyme of the invention.

Moreover, the invention relates to the use of a xylanase of the invention in processes for treatment of lignocellulosic pulp.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the relative activity (% rel.) of a protease of the invention at various pH, determined at 25°C with casein as substrate; and

Fig. 2 shows the relative activity (% rel.) of a protease of the invention at various temperatures (■ Buffer pH 9.5; □ Buffer pH 9.5 containing 0.1% STPP), determined at pH 9.5 with casein as substrate.

DETAILED DISCLOSURE OF THE INVENTION

The Microorganism

The present invention relates to microorganisms of the novel alkalophilic species Bacillus sp. AC13, represented by the type culture Bacillus sp. AC13, NCIMB 40482.

The strain Bacillus sp. AC13, NCIMB 40482, has been deposited on 3 March 1992 according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, at National Collections of Industrial and Marine Bacteria Ltd., 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, UK, under Accession No. NCIMB 40482.

The microorganisms of this invention are aerobic, rod shaped, spore forming bacteria, and therefore belonging to the genus Bacillus.

Morphologically they can be described as rods having a diameter of 0.6-0.9 μm and a length of 4-8 μm. The spores are ellipsoid to round, approximately 1.2 x 0.8 μm, terminal swelling the sporangia, giving the sporangium a characteristic racket or drumstick like shape.

The microorganisms of Bacillus sp. AC13 are obligately alkalophilic, requiring carbonate buffer pH 9 to 10 in the agar media for growth. Optimal growth is observed at 37°C, at pH 9.5-10. No growth at 50°C and no growth at pH 7.

Colonies on potato dextrose agar (Difco™) added 0.1 M sodium sesquicarbonate are white with characteristic dendroid to hairy edges.

The microorganisms can be further described by the following characteristics.

NaCl tolerance weak growth at 12%	0-10%	
Growth temperature no growth at 50°C	≤ 45°C	
Hydrolysis of	casein	positive
	gelatine	positive
	pullulan	negative
	starch	positive
	cellulose	positive
	xylan	positive
Catalase reaction	positive	
Aminopeptidase test	negative	
Deamination of phenylalanine	negative	

Reduction of nitrate	positive
Major fatty acids	C 15:0 ISO (≈ 45%)
	C 15:0 ANTEISO (≈ 25%)
	ISO 17:1 w10c (≈ 10%)
	Unsaturated: 20%
	Branched approx. 50%

Cultivation of the Microorganism

The microorganism of the invention can be cultivated under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art.

Suitable carbon sources are carbohydrates such as sucrose, glucose and starch, or carbohydrate containing materials such as cereal grain, malt, rice and sorghum. The carbohydrate concentration incorporated in the medium may vary widely, e.g. up to 25% and down to 1 - 5%, but usually 8 - 10% will be suitable, the percentages being calculated as equivalents of glucose.

The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources quite a number are used regularly in fermentation processes involving the cultivation of bacteria. Illustrative examples are soybean meal, cotton seed meal, peanut meal, casein, corn, corn steep liquor, yeast extract, urea and albumin. In addition, the nutrient medium should also contain usual trace substances.

Since the novel Bacillus species of this invention are alkalophilic and unable to grow at pH below 7, the cultivation is preferably conducted at alkaline pH values, which can be obtained by addition of suitable buffers such as sodium carbonate or mixtures of sodium carbonate and sodium bicarbonate, after sterilization of the growth medium. For cultivation in tank fermentors it is necessary to use artificial aeration. The rate of aeration is similar to that used in conventional tank fermentation.

After fermentation, liquid enzyme concentrates may be produced by removal of coarse material from the broth or, if desired, concentration of the broth by evaporation at low temperature or by reverse osmosis. Finally, preservatives may be added to the concentrate.

Solid enzyme preparations may be prepared from the purified and/or concentrated broth by precipitation with salts, such as Na_2SO_4 or water-miscible solvents, such as ethanol or acetone. Removal of the water in the broth by suitable drying methods such as spray-drying may also be employed.

The microorganisms of the invention have been found to be able to produce valuable novel enzymes, in particular proteases, xylanases and cellulases.

The Enzymes

The enzymes of the invention are obtainable by cultivation of a microorganism of the invention, preferably Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts. The enzymes may also be obtained by recombinant DNA-technology.

In more specific aspects, the enzymes of the present invention can be further described by the following physical-chemical characteristics.

The Proteases

In a specific embodiment of the invention, a protease can be further characterized by having an apparent molecular weight of approximately 30 kD as determined by SDS-PAGE, and a pI of approximately 9.3 as determined by isoelectric focusing on LKB Ampholine PAG plates.

The protease can also be characterized by having proteolytic activity at pH values of from below pH 6 to above pH 11, having optimum above pH 10, around pH 11, when determined at 25°C with casein as substrate.

Moreover, the protease can be characterized by having proteolytic activity at temperatures of from approximately 15°C to above 70°C, having activity optimum at temperatures in the

range 45-55°C, around 50°C, when determined at pH 9.5 with casein as substrate. This activity optimum can be detected with or without sodium tripolyphosphate, which is a common ingredient in many commercial detergents.

The Xylanases

In a specific embodiment of the invention, a xylanase can be further characterized by having an apparent molecular weight of approximately 25 kD when determined by SDS-PAGE, and a pI of approximately 9 when determined by isoelectric focusing on LKB Ampholine PAG plates.

The Cellulases

In a specific embodiment of the invention, two cellulases can be further characterized, one by having an apparent molecular weight of approximately 45 kD when determined by SDS-PAGE, and a pI of approximately 4.3 when determined by isoelectric focusing on LKB Ampholine PAG plates, and one by having an apparent molecular weight of approximately 55 kD when determined by SDS-PAGE, and a pI of approximately 4.5 when determined by isoelectric focusing on LKB Ampholine PAG plates.

Immunochemical Properties

The enzymes of the invention have immunochemical properties identical or partially identical (i.e. at least partially identical) to those of an enzyme derived from the strain Bacillus sp. AC13, NCIMB No. 40482.

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to Axelsen N.H.; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, chapters 5, 19 and 20.

Monospecific antisera are generated according to the above mentioned method by immunizing rabbits with the purified enzymes of the invention. The immunogens are mixed with Freund's adjuvant and injected subcutaneously into rabbits every second week. Antisera are obtained after a total immunization period of 8 weeks, and immunoglobulins are prepared therefrom as described by Axelsen N.H., supra.

Assay for Proteolytic Activity

The proteolytic activity is determined with casein as substrate.

One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 mM of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

A folder AF 228/1 describing the analytical method is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Assay for Xylanolytic Activity

The xylanolytic activity is measured in endo-xylanase units (EXU), determined at pH 9.0 with remazol-xylan as substrate.

A xylanase sample is incubated with remazol-xylan substrate. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is proportional to the xylanase activity, and the xylanase units are then determined relatively to an enzyme standard at standard reaction conditions, i.e. at 50.0 +/- 0.1°C, pH 9.0, and 30 minutes' reaction time.

A folder AF 293.9/1 describing the analytical method is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Assay for Cellulytic Activity

The cellulytic activity is measured in cellulase viscosity units (CEVU), determined at pH 9.0 with carboxymethyl cellulose (CMC) as substrate.

Cellulase viscosity units are determined relatively to an enzyme standard (< 1% water, kept in N_2 atmosphere at -20°C; arch standard at -80°C). The standard used, 17-1187, is 4400 CEVU/g under standard incubation conditions, i.e. pH 9.0, Tris Buffer 0.1 M, CMC 7 LFD substrate 33.3 g/l, 40.0°C for 30 minutes.

A folder AF 253/1 describing the analytical method is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Industrial Applications

The enzymes of this invention possess valuable properties allowing for various industrial applications. In particular the proteases and cellulases of the invention, in being alkaline, find potential application in e.g. detergent compositions. The cellulases may find potential application in the textile industry, e.g. for Bio-Polishing. The xylanases may find application in e.g. the paper pulp industry.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Cultivation Example

The strain Bacillus sp. AC13, NCIMB 40482, was cultivated at 30°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of medium of the following composition (per litre):

Potato starch	100	g
Ground barley	50	g
Soybean flour	20	g
Na_2HPO_4 x 12 H_2O	9	g
Pluronic®	0.1	g
Sodium caseinate	10	g

The starch in the medium is liquified with α-amylase and the medium is sterilized by heating at 120°C for 45 minutes.

After sterilization the pH of the medium is adjusted to 9.7 by addition of 10 ml 1 M sodium sesquicarbonate to each flask.

After 3 days of incubation the following activities were observed:

Proteolytic activity	20	CPU/l
Xylanolytic activity	10	EXU/g
Cellulytic activity	4	CEVU/g

Isoelectric focusing on gels overlayered with different substrates at least 2 different proteases, at least 4 different xylanases, and at least 2 different cellulases were detected.

The major proteolytic band has a pI of approximately 9.3 and a molecular weight of approximately 30 kD.

The major xylanolytic band has a pI of approximately 9 and a molecular weight of approximately 25 kD.

The major cellulytic band has a pI of approximately 4.3 and a molecular weight of approximately 45 kD.

EXAMPLE 2

Purification of the Proteolytic Compounds

After cultivation, the proteolytic activity of the fermentation broth of Ex. 1 was found to be 20 CPU/l. In this fermentation broth at least two proteolytic enzymes have been identified by isoelectric focusing on LKB Ampholine PAG plates.

After separation of the solid material, the major proteolytic component was purified by a conventional chromatographic method. From 1 litre of culture broth yield was 50 ml of protease preparation with a proteolytic activity of 236 CPU/l (60%).

The purified protease has an apparent pI value of 9.3 when determined by isoelectric focusing on LKB Ampholine PAG plates. By SDS-PAGE the apparent molecular weight of the protease is found to be 30 kD. Purity was more than 90% as judged by both SDS-PAGE and isoelectric focusing.

The activity was determined using the assay for proteolytic activity described above. The results of these experiments are presented on the appended Figs. 1-2.

EXAMPLE 3

Purification of the Xylanolytic Compounds

In the fermentation broth, as obtained according to Ex. 1, at least four xylanolytic enzymes have been identified by isoelectric focusing combined with an overlayer of xylan. The xylanolytic enzymes cover the pI range of from 5 to 9.5.

From the fermentation broth of Ex. 1, a xylanase with an alkaline pI (the major xylanolytic component) was purified to homogeneity by conventional chromatographic techniques involving cation exchange chromatography on S-Sepharose High Load™ and Mono S™, hydrophobic adsorption chromatography on Phenyl-Sepharose, as well as affinity chromatography to specific removal of proteinases.

The purified xylanase has an apparent pI value of 9 in a 3.5 to 9.5 isoelectric focusing gel. By SDS-PAGE the apparent molecular weight of the xylanase is found to be 25 kD.

EXAMPLE 4

Purification of the Cellulytic Compounds

In the fermentation broth obtained according to Ex. 1 at least two cellulytic enzymes have been identified by isoelectric focusing.

The fermentation broth of Ex. 1 was filtrated and applied on a cellulase affinity column. After wash at pH 8.5 in Tris buffer, the column was eluted at high pH 11.8 with triethylamine. The pH in the eluate was adjusted to 7.5 and UF-concentrated and washed out with Tris buffer.

The concentrate was applied on a Mono Q™ column (Pharmacia) and eluted with a linear gradient with 15 column volumes in Tris buffer pH 9.0 with 0.5 M NaCl.

The cellulase containing fractions were subjected to isoelectric focusing and SDS-PAGE, and two cellulytic components were found, one having a MW of approx. 45 kD and a pI of approx. 4.3, and one having a MW of approx. 55 kD and a pI of approx. 4.5.

EXAMPLE 5

N-Terminal Amino-Acid Analysis

The N-terminal amino-acid sequence of the cellulase, having a MW of approx. 45 kD, obtained according to Ex. 4, was determined using standard methods for obtaining and sequencing peptides [Findlay & Geisow (Eds.), Protein sequencing - a practical approach, 1989, IRL Press].

The N-terminal amino-acid sequence was found to be (SEQ ID No. 1 of the attached sequence listing):

Asp-Xaa-Asp-Xaa-Val-Val-Glu-Glu-His-Gly-Gln-Leu-Arg-Ile-Xaa-Asn-Gly-Xaa-Leu.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: 45 44 44 88 88
(H) TELEFAX: 44 49 32 56
(I) TELEX: 37304

(ii) TITLE OF INVENTION: NOVEL MICROORGANISMS

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus sp.
(B) STRAIN: AC 13 NCIMB 40482

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Xaa Asp Xaa Val Val Glu Glu His Gly Gln Leu Arg Ile Xaa Asn
1 5 10 15

Gly Xaa Leu

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 1, line 7 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

NATIONAL COLLECTIONS OF INDUSTRIAL & MARINE BACTERIA LTD.

Address of depositary institution (including postal code and country) [4]

23 St. Machar Drive, Aberdeen AB2 1RY, Scotland

Date of deposit [5]	Accession Number [6]
9 March 1992	NCIMB 40482

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited micro-organism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Anne-Grethe Henriksson
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. An isolated biologically pure culture of a strain of Bacillus sp. AC13.

2. A culture according to claim 1, the strain being Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof.

3. An enzyme obtainable from a strain of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of an enzyme derived from Bacillus sp. AC13, NCIMB No. 40482.

4. An enzyme according to claim 3, being obtainable from the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof.

5. A protease obtainable from a strain of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a protease derived from Bacillus sp. AC13, NCIMB No. 40482.

6. A protease according to claim 5, further characterized by:

(a) An apparent molecular weight of approximately 30 kD as determined by SDS-PAGE;

(b) A pI of approximately 9.3 as determined by isoelectric focusing on LKB Ampholine PAG plates;

(c) Activity optimum above pH 10 determined at 25°C with casein as substrate; and

(d) Activity optimum at temperatures in the range 45-55°C, around 50°C, determined at pH 9.5 with casein as substrate.

7. A protease according to either of claims 5-6, being obtainable from the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof.

8. A xylanase obtainable from a strain of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a xylanase derived from Bacillus sp. AC13, NCIMB No. 40482.

9. A xylanase according to claim 8, further characterized by:

(a) An apparent molecular weight of approximately 25 kD as determined by SDS-PAGE; and

(b) A pI of approximately 9 as determined by isoelectric focusing on LKB Ampholine PAG plates.

10. A xylanase according to either of claims 8-9, being obtainable from the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof.

11. A cellulase obtainable from a strain of Bacillus sp. AC13, and having immunochemical properties identical or partially identical to those of a cellulase derived from Bacillus sp. AC13, NCIMB No. 40482.

12. A cellulase according to claim 11, further characterized by:

(a) An apparent molecular weight of approximately 45 kD as determined by SDS-PAGE;

(b) A pI of approximately 4.3 as determined by isoelectric focusing on LKB Ampholine PAG plates; and

(c) A N-terminal amino-acid sequence identified by ID No. 1 of the attached sequence listing.

13. A cellulase according to claim 11, further characterized by:

(a) An apparent molecular weight of approximately 55 kD as determined by SDS-PAGE; and

(b) A pI of approximately 4.5 as determined by isoelectric focusing on LKB Ampholine PAG plates.

14. A cellulase according to any of claims 11-13, being obtainable from the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof.

15. A process for the preparation of an enzyme according to any of claims 3-14, which process comprises cultivation of a strain of Bacillus sp. AC13, preferably the strain Bacillus sp. AC13, NCIMB No. 40482, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme.

16. A detergent additive comprising a protease according to any of claims 5-7, and/or a cellulase according to any of claims 11-14, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

17. A detergent composition comprising a protease according to any of claims 5-7 and/or a cellulase according to any of claims 11-14.

18. A detergent composition according to claim 17, which further comprises one or more other enzymes, in particular lipases, amylases, cellulases, oxidases and/or peroxidases.

19. The use of the xylanase according to any of claims 8-10 in a process for treatment of lignocellulosic pulp.

20. A process according to claim 19 for treatment of lignocellulosic chemical pulp, wherein the lignocellulosic pulp is treated with the xylanase at a pH above 6.5, preferably above 7.5, whereafter the thus treated cellulosic pulp is treated with chlorine at an active chlorine multiple of 0.20 or less in the first chlorination stage.

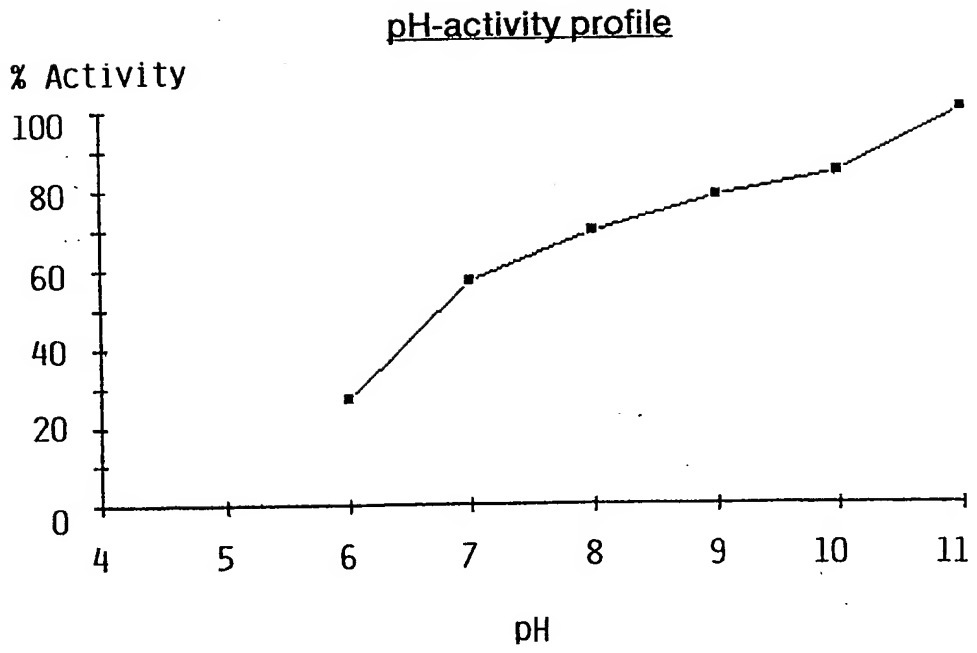

Fig. 1

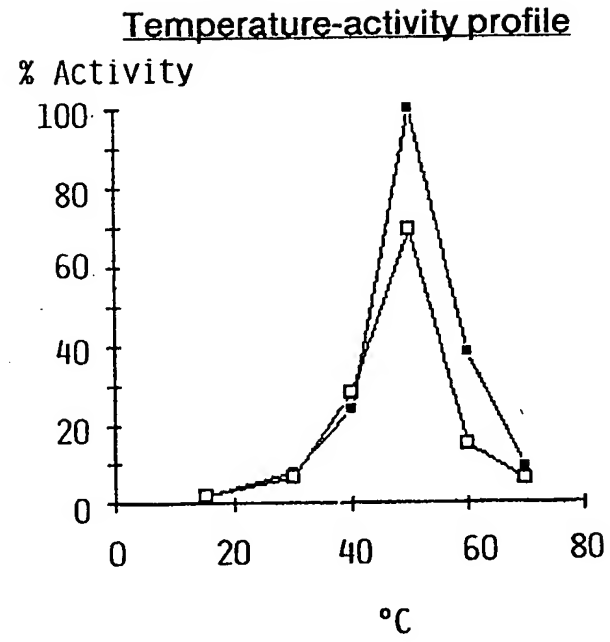
Temperature-activity profile
% Activity
100
80
60
40
20
0
0
20
40
60
80
°C

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00218

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 1/20, C12N 9/54, C12N 9/42, C12N 9/24 // (C 12 N 1/20, C 12 R 1:07), C 11 D 3/386, D 21 C 9/00, C 12 S 3/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 9217577 (NOVO NORDISK A/S), 15 October 1992 (15.10.92), see claim 1 --	1-2,5-7, 15-18
P,X	WO, A1, 9217578 (NOVO NORDISK A/S), 15 October 1992 (15.10.92), see claim 1 --	1-2,5-7, 15-18
P,X	WO, A1, 9217576 (NOVO NORDISK A/S), 15 October 1992 (15.10.92), see claim 1 --	1-2,5-7, 15-18
X	US, A, 4480037 (FIJI ICHISHIMA ET AL), 30 October 1984 (30.10.84), see claim 1 --	1-2,5-7, 15-18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1993	15 -12- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00218

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE, C2, 3328619 (SHOWA DENKO K.K.), 17 Sept 1992 (17.09.92), see claim 1	1-2,5-7, 15-18
X	Dialog Information Services, file 357, Biotechnology Abstract, 82-92, Dialog accession no. 092501, DBA accession no.89-10492, LION: "Production of a novel alkaline protease-L-isolation from Bacillus sp. and characterization", Patent number: JP 1101884, Patent Date: 890419	1-2,5-7, 15-18
X	US, A, 4771003 (EDMUND J. STELLWAG ET AL), 13 Sept 1988 (13.09.88), see claim 7	1-2,5-7, 15-18
X	US, A, 4764470 (DONALD R. DURHAM ET AL), 16 August 1988 (16.08.88), see claim 6	1-2,5-7, 15-18
A	Patent Abstracts of Japan, Vol 13,No 302, C-616, abstract of JP, A, 1-91772 (NIKKO BIO GIKEN K.K. et al), 11 April 1989 (11.04.89)	1
X	WO, A1, 9118978 (VALTION TEKNILLINEN TUTKIMUSKESKUS), 12 December 1991 (12.12.91), see page 3, line 14-22 and abstract	8-10,15, 19-20
X	WO, A1, 9203540 (NOVO NORDISK A/S), 5 March 1992 (05.03.92), see page 13 example 6 and claim 4	8-10,15, 19-20
X	EP, A2, 0339550 (KAO CORPORATION), 2 November 1989 (02.11.89), see claim 1 and abstract	11-15
X	WO, A1, 9110732 (NOVO NORDISK A/S), 25 July 1991 (25.07.91), see abstract and claim 8	11-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00218

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0468464 (SHOWA DENKO KABUSHIKI KAISHA), 29 January 1992 (29.01.92), see the abstract --	11-15
A	EP, A2, 0270974 (KAO CORPORATION), 15 June 1988 (15.06.88), see page 85 --	11-15
A	Patent Abstracts of Japan, Vol 13,No 336, C-623, abstract of JP, A, 1-112982 (KAO CORPORATION ET AL), 1 May 1989 (01.05.89) -- --------	11-15

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/DK 93/00218

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3,4,8,10,11,14
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See next sheet!

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See next sheet!

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 93/00218

Box II Observations where unity of inventions is lacking

I Claims 1-2, 5-7, 16-18 and part of claim 15 directed to a strain of Bacillus sp. AC13, a protease obtainable therefrom, a process for its preparation and a detergent additive.

II Claims 8-10, 19-20 and part of claim 15 directed to a xylanase obtainable from a strain of Bacillus sp. AC13, a process for its preparation and uits use.

III Claims 11-14 and part of claim 15 directed to a cellulase obtainable form a strain of Bacillus sp. AC13, and a process for its preparation.

INTERNATIONAL SEARCH REPORT

International application No.

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Box I Observations where certain claims were found unsearchable

The wordings of claims 3, 4, 8, 10, 11, 14 are too broadly formulated to permit a meaningful search. The expression "enzymes from Bacillus sp. AC13 having immunochemical properties identical or partially identical to ....." is such a broad expression that it includes a vaste number of different enzymes including already known enzymes. Even if the word "partially" would be omitted the claims would still be to broadly formulated to be searched.
The search has therefore been incomplete (see Art 6).

INTERNATIONAL SEARCH REPORT

Information on patent family members

16/10/93

International application No.
PCT/DK 93/00218

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9217577	15/10/92	NONE		
WO-A1- 9217578	15/10/92	NONE		
WO-A1- 9217576	15/10/92	NONE		
US-A- 4480037	30/10/84	AU-B-	556045	23/10/86
		AU-A-	1097083	18/08/83
		CA-A-	1193213	10/09/85
		FR-A,B-	2521163	12/08/83
		GB-A,B-	2116561	28/09/83
		JP-C-	1614949	15/08/91
		JP-A-	58134990	11/08/83
		JP-B-	60055118	03/12/85
DE-C2- 3328619	17/09/92	BE-A-	897479	01/12/83
US-A- 4771003	13/09/88	EP-A-	0220921	06/05/87
US-A- 4764470	16/08/88	EP-A-	0232169	12/08/87
WO-A1- 9118978	12/12/91	AU-A-	7990391	31/12/91
WO-A1- 9203540	05/03/92	CA-A-	2090337	25/02/92
		EP-A-	0546045	16/06/93
EP-A2- 0339550	02/11/89	JP-A-	1273587	01/11/89
		US-A-	5045464	03/09/91
WO-A1- 9110732	25/07/91	AU-A-	7189491	05/08/91
		EP-A-	0510091	28/10/92
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		US-A-	5231022	27/07/93
EP-A2- 0270974	15/06/88	JP-A-	63273474	10/11/88
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		JP-A-	63273475	10/11/88
		JP-A-	63279790	16/11/88
		JP-A-	1037286	07/02/89
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		JP-A-	63141586	14/06/88

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